EVIDENCE FOR A SWARMING SUBSTANCE[1] WHICH STIMULATES COLONY FORMATION IN THE DEVELOPMENT OF PEDIASTRUM DUPLEX MEYEN[2]

JOHN G. MONER

Department of Biology, Princeton University, Princeton, New Jersey[3]

The life cycle of the colonial, green alga *Pediastrum* has been known since the studies of Braun (1855), Askenasy (1888), Smith (1916) and Harper (1913, 1916, 1918a, 1918b). The colonies or coenobia of this organism are commonly found in fresh water and are disk-shaped in form. The cells of the colony are arranged in a single layer with the marginal cells usually differing from those of the interior by possessing one or two prongs or processes produced radially outward. The number of cells in a colony varies but in general, can be described by the simple formulation 2^N, the common range being eight to sixty-four. Asexual reproduction involves the formation of biflagellate zoospores which arise through successive nuclear divisions, followed by progressive cleavage of the cell chloroplast. After the zoospores have been formed they are discharged through a crescent-shaped slit in the mother cell wall and are enclosed in a thin, transparent sac or vesicle. At first, they form an irregularly shaped mass, but after three to four minutes of active movement, they slow down forming a flat plate of cells. Complete cessation of movement then follows and prong formation begins and is completed in a matter of minutes.

Although details of the life cycle have long been known, little work has been done concerning the factors affecting the production of zoospores and their release to form new colonies. The purpose of the study presented in this paper was to examine the characteristics of growth and reproduction in *Pediastrum* when grown in culture and to investigate the role of certain factors which influence these processes. The results will be concerned with such aspects as the time at which colony formation begins and how its initiation is affected by pH and aged medium. Evidence will be presented for the occurrence of a substance or group of substances in aged medium which can alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell number.

Materials and Methods

The species of *Pediastrum* used in this study was *P. duplex* Meyen (from Malham) and was provided by E. G. Pringsheim of the Cambridge University Botany School in unialgal, non-bacteria-free culture. It was grown in this form

[1] The word "substance" is used to denote one or more substances active in stimulating colony formation in *Pediastrum duplex* Meyen.

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[3] Present address: Tropical Research Medical Laboratory, A.P.O. 851 C/O Postmaster, New York, New York.

on mineral medium No. 8 according to Chu (1942) with 10% soil extract added. All of the cultures used in this section were prepared under sterile conditions and grown in Ehrlenmeyer flasks.

For most of the experiments the culture vessels were placed in a trough with tap water circulating through it to maintain a relatively constant temperature. The lighting was provided by two 15-watt daylight General Electric "fluorescent" bulbs set parallel 26 cm. apart in a wooden housing and elevated 25 cm. above the culture-containing trough. In one experiment only (Section A), the culture was not grown in a water-circulated trough but was simply placed on a wooden platform and allowed to grow at room temperature (24–28° C.). This culture was illuminated by a 100-watt tungsten filament bulb housed in a desk-type lamp and placed 40 cm. in front of the culture. In all cases the lighting was continuous throughout the course of the experiment. It should be noted that temperature was not continuously recorded by any mechanical device but that periodic measurements were made daily using a standard mercury thermometer. When the cultures were grown in the water-circulated trough the temperature of the circulating water was determined; when a culture was grown in the open air the temperature of a flask of water placed next to the culture flask was determined. The constancy of the light source (*i.e.*, intensity and wave-length) was also not strictly controlled. It was felt by the author that the aim of this investigation did not necessitate the use of rigid controls of temperature and light since in every experiment both control and experimental cultures were grown under identical conditions. Any investigation of the effects of temperature and light on the phenomena reported in this paper would, of course, require strict control of these two factors and it is hoped that a study of this nature will be possible during the course of future work.

For counting the number of colonies in a culture, two counting chambers were used: The Levy hemacytometer and the Sedgwick-Rafter counting chamber. For determining colony number with the Levy hemacytometer the method for counting leucocytes was used according to the following formulation:

$$\text{Colonies/cc.} = \frac{\text{Colonies counted} \times \text{dilution} \times 10^4}{\text{No. of 1 sq. mm. areas counted}}$$

To determine colony number with the Sedgwick-Rafter counting chamber the number of colonies in one pathway was counted using a 10 × objective and 20 × ocular and the following formulation used:

$$\text{Colonies/cc.} = 17 \times \text{colonies in one pathway} \times \text{dilution} \times 2.$$

The colonies were always fixed in 2–3% formalin before counting. In all experiments but one the Sedgwick-Rafter chamber was used to determine colony number.

To determine cleavage number the colonies were fixed in formalin-aceto-alcohol and stained with Harris' hematoxylin according to the method of Johansen (1940). A drop of suspension containing stained colonies was then placed under a cover slip, sealed with paraffin and the number of cells showing cleavage figures was recorded for 150 colonies counted. To make these counts the 95 × oil immersion objective and 10 × ocular were used. pH measurements were made with a Cambridge pH-Meter Laboratory Model-L.

Results

A. Quantitative relations of growth and colony production

At the outset of this work on *Pediastrum* it was desirable to obtain large numbers of swarming colonies in a relatively short time. To accomplish this, large inocula of 5 cc. for every 50 cc. of fresh medium were used in preparing cultures. When this was done it was noticed that swarming occurred in great numbers at a specific time after inoculation and that it seemed to last for only a few days. The time that swarming or colony production began could be predicted with reasonable accuracy in succeeding cultures inoculated in the same manner from the same stock. Virtually no swarming was found in the cultures prior to this swarming period and after it was completed.

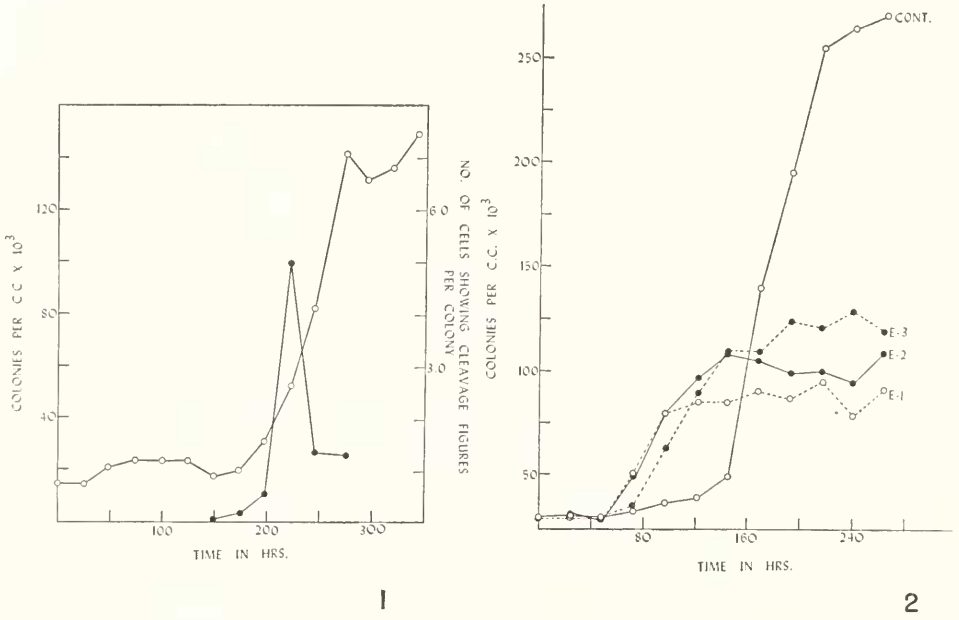


Figure 1. Graph showing colony production and cleavage activity curves. Open circles represent colony production and black dots represent cleavage activity.

Figure 2. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 1 (E-2) and 2 (E-3) days' growth in fresh or control medium.

In order to obtain a quantitative description of colony formation as a function of time the following experiment was performed: a culture was started consisting of 50 cc. of fresh medium inoculated with a 5-cc. suspension of 99-day old stock culture and grown in a 125-cc. flask. The lighting was provided by a 100-watt tungsten filament bulb and the experiment run at room temperature (24–28° C.; see Materials and Methods). One-cc. aliquots were removed daily from the culture, fixed and colony number determined using a Levy hemacytometer chamber. In the results in Figure 1 it is seen that no colony production occurs in the culture up to 173 hours but that a sudden burst begins at 197 hours and lasts for 78 hours at

which time it begins to level off. The final number of colonies is approximately 8 times the value of the starting colonies which is what would be expected if the original mean colony size was 8 cells (see Discussion). Cleavage counts were made to see whether cleavage activity coincided with the burst phase of the curve. In the graph of Figure 1 this activity is expressed as the number of cells showing cleavage figures per colony and it can be seen that there is a correlation between colony formation and cleavage activity. It should be noted that although no swarming takes place in the culture up to 173 hours, considerable growth, in terms of colony size, occurs during this period.

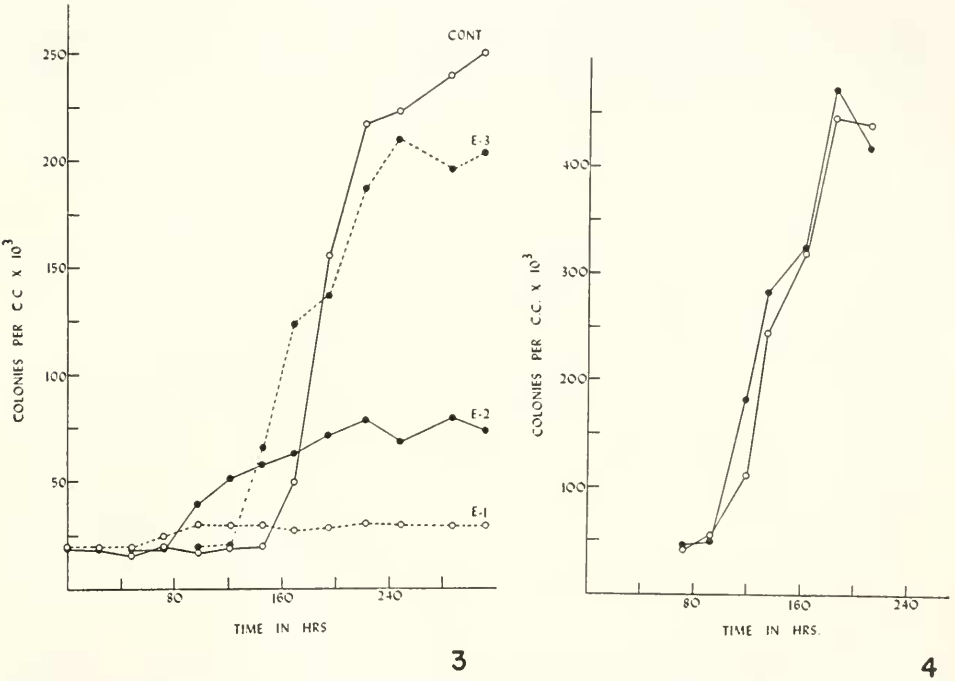


FIGURE 3. Graph showing colony production in fresh or control medium and in aged medium after 0 (E-1), 2 (E-2) and 4 (E-3) days' growth in fresh or control medium.

FIGURE 4. Graph showing results obtained when colonies are placed in aged medium one day prior to the time they will swarm in fresh or control medium. Open circles represent colony production in the control and black dots represent colony production in the experimental.

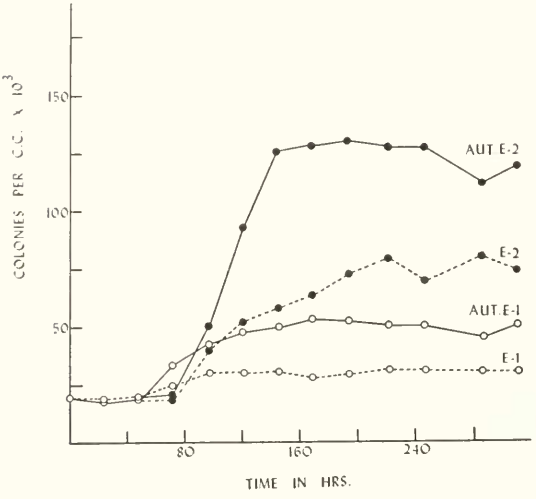
B. Evidence for a substance in the aged medium affecting colony reproduction

The curious nature of the swarming curve suggested the possibility of some change occurring in the medium which might initiate swarming or colony formation. Experiments were performed to test how colony production was affected by moderately aged medium, in which swarming had occurred. In the first experiment 150 cc. of fresh medium were inoculated with 15 cc. of a 53-day old stock culture and placed in a 500-cc. flask to serve as standard culture. Aged medium was obtained from a 19-day old culture from which the colonies had been removed

by centrifugation. Using the standard culture and aged medium the following cultures were prepared:

Control: 30 cc. of standard culture. Prepared at zero time.
Experimental No. 1: Colonies from 30 cc. of standard culture, fresh medium removed and replaced by 30 cc. of aged medium. Prepared at zero time.
Experimental No. 2: Colonies from 30 cc. of standard culture after one day's growth, fresh medium removed and replaced by 30 cc. aged medium.
Experimental No. 3: Colonies from 30 cc. of standard culture after two days' growth, fresh medium removed and replaced by 30 cc. aged medium.

One-cc. aliquots were removed daily from all the cultures and colony number determined. The temperature maintained throughout the course of the experiment was $20 \pm 2°$ C. In the results shown in Figure 2 it is seen that the control shows



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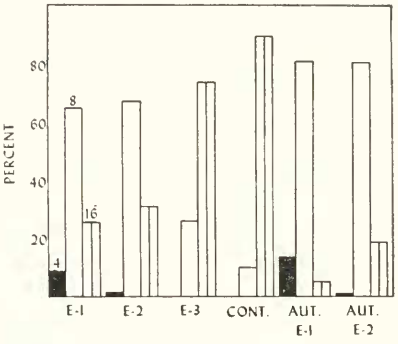

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FIGURE 5. Graph showing results obtained when colonies are grown in unautoclaved and autoclaved aged medium after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium.
FIGURE 6. Graph showing the final percentage distribution of four-, eight- and sixteen-celled colonies produced in the control and the unautoclaved and autoclaved aged medium cultures of Figures 3 and 5.

the familiar swarming burst after 144 hours but that all three experimentals show a substantial increase in colony number after 73 hours. The longer the colonies grow in fresh medium, the greater the level of colony production reached after they are transferred to aged medium. None of the experimental cultures produce as many new colonies as the control.

In a repeat experiment similar to the preceding one colonies were allowed to grow in fresh or control medium for 0 (Exp. No. 1), 2 (Exp. No. 2) and 4 (Exp. No. 3) days before being transferred to aged medium. The standard culture was prepared from a 58-day old stock culture and the aged medium was obtained from

a 15-day old culture. In the results shown in Figure 3 it is apparent that all three experimental cultures show colony production before the control does. Once again, the longer the colonies grow in fresh or control medium the greater the number of new colonies they produce when transferred to aged medium. The colonies grown for 4 days in fresh or control medium prior to transfer to aged medium show a final level of colony production very close to that of the control.

Figure 4 shows the results obtained when colonies, one day prior to the time they will normally begin swarming, are placed in 8-day old aged medium. In this experiment the colonies had been growing three days in fresh medium when they were placed in aged medium. The standard culture was prepared using 87-day old stock culture and the temperature maintained throughout the course of the experiment was $19 \pm 2°$ C. It is seen that although both cultures begin swarming at the same time the experimental shows a greater burst. Essentially the same final level of colony production is reached in both cultures.

TABLE I

Final distribution of colony types produced in the control and experimental cultures of Figures 3 and 5.

Culture	4-celled colonies		8-celled colonies		16-celled colonies	
	New colonies/cc.	% of total	New colonies/cc.	% of total	New colonies/cc.	% of total
E-1	1,112	8.52	8,588	65.8	3,356	25.7
E-2	756	1.52	33,568	67.6	15,331	30.8
E-3	0	0	50,837	26.0	144,983	74.0
Control	0	0	20,624	9.95	186,497	90.0
Aut. E-1	4,427	13.9	25,826	81.2	1,571	4.94
Aut. E-2	1,163	1.17	80,294	80.5	18,403	18.4

C. The effect of high temperature on the activity of the aged medium

To test the effect of high temperature on the activity of aged medium the following experiment was performed: Aged medium used in the second experiment reported in section B was autoclaved for 30 minutes under 15 lbs./in.^{2} pressure at 121° C. Colonies were placed in this autoclaved aged medium after 0 and 2 days growth in fresh medium and the cultures were run simultaneously with Experimentals No. 1 and No. 2 of the second experiment reported in section B, both of which contained unautoclaved, aged medium. From the results shown in Figure 5 it is evident that the two autoclaved experimentals show colony production at the same time as the unautoclaved counterparts. However, the colonies placed in autoclaved aged medium after 0 days growth in fresh medium produce 3 times as many new colonies as the unautoclaved partner, while those placed in autoclaved aged medium after two days' growth in fresh medium produce almost twice as many new colonies as the unautoclaved partner.

Table I and Figure 6 show the per cent distribution of 4-, 8- and 16-celled colonies produced by the control and three experimental cultures of the second experiment reported in section B and the two autoclaved aged medium experi-

mentals of this section. The results show that the longer the colonies are kept in the control medium before transfer to aged medium, the greater the tendency to produce colonies with larger cell number.

D. The role of pH of the medium

Two experiments were performed to determine whether the effect of aged medium in causing premature colony formation was due to pH change in the medium. Since the medium is poorly buffered it was thought that pH change

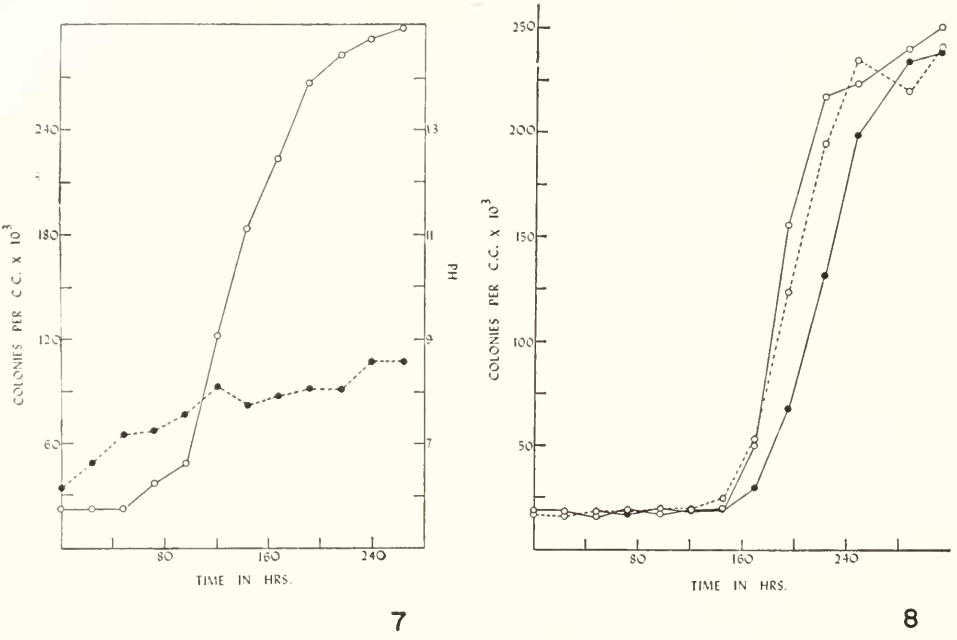

FIGURE 7. Graph showing curves of colony production and pH change of the medium during colony production. Open circles represent colony production and black dots represent pH change.

FIGURE 8. Graph showing results obtained when colonies are grown in pH-regulated medium (7.31) after 0 (E-1) and 2 (E-2) days' growth in fresh or control medium. Open circles connected by continuous line represent colony production in the control; open circles connected by dotted line represent colony production in pH-regulated E-1 and black dots represent colony production in pH-regulated E-2.

might be an influencing factor. A culture was started consisting of 200 cc. of fresh medium inoculated with 20 cc. of 56-day old stock culture and grown in a 500-cc. flask. Two (5-cc.) aliquots were removed daily and pH and colony number determinations made. The temperature maintained throughout the course of the experiment was $19 \pm 2°$ C. Figure 7 shows the results obtained. It is clear that change of pH from the acid to the basic side occurs during the growth and reproduction of *Pediastrum* under these culturing conditions; pH changed from an initial 6.13 to a final 8.60 but there does not appear to be any sudden increase in pH

at the time of colony formation. Instead a gradual, continuous increase occurred both prior to and during this phase.

Since pH change did occur in the medium during the development of *Pediastrum* another experiment was performed to test whether this change was the causative influence in the premature swarming effect of aged medium. In this experiment th pH of fresh medium (6.29) was regulated with 0.1 N NaOH to that of the aged medium used in the second experiment of section B (7.31). Colonies were placed in this pH-regulated medium after 0 and 2 days' growth in fresh, unregulated medium and the culture was run simultaneously with the control and Experimental No. 1 and No. 2 of the second experiment of section B. In the results shown in Figure 8 it is seen that both pH-regulated cultures show the swarming burst at approximately the same time as the control. The colonies receiving the pH-regulated medium after two days' growth in fresh unregulated medium apparently respond somewhat later than those of the control. When the experiment was completed the pH of the control was 7.51, the 2-day aged medium experimental (Exp. No. 2) was 7.50 and the 2-day pH regulated experimental was 7.72.

Discussion

It has been shown for the first time that colonies of *Pediastrum* when grown in cultures using large inocula in fresh media exhibit two developmental phases. The first is a latent period which may last for 3–7 days, in which the colonies grow but do not reproduce. The second phase is a very active period of swarming or colony production which lasts 3–4 days during which all the cells in the initial inoculum give rise to new colonies. Cleavage activity, as would be expected, has been shown to be quite synchronous with this period. That essentially all the cells in the original colonies give rise to new colonies can be demonstrated by determining the average number of cells per colony in the inoculum and multiplying this value by the number of colonies present. In the experiment concerning colony type distribution the control showed an average colony size of 14.0 cells/colony and a colony number of 18,718/cc. in the starting culture. If all the cells gave rise to colonies then the value reached by the control after swarming should be (14.0) (18,718) or 262,052/cc. The actual value reached by the control is reasonably close: 252,450/cc.

The fact that cultures exhibit a latent period might have initially been interpreted as some inherent time lag in the ability of the colonies to reproduce during this period. However, the results show that by placing colonies in medium in which swarming has occurred colony formation can be induced prematurely. The longer the preliminary period of growth in control or fresh medium, the greater the number of colonies produced after transfer to aged medium and the more closely the resulting swarming curve resembles that of the control. Coincident with these changes is the gradual increase in colony size in terms of cell number as the preliminary period of growth in control or fresh medium is lengthened. Again, the longer this preliminary period of growth the more closely the colony type distribution resembles that of the control. It appears evident that the latent period in the normal swarming curve of *Pediastrum* is one in which the competence of the colonies to reproduce is ever increasing. This might conceivably be a function of critical size whereby colonies which are too small and immature to reproduce are

transformed, during the period of growth, into colonies of adequate size. The longer this period of growth the more abundant the number of colonies reaching reproductive maturity. The fact that colonies can reproduce during the latent period, and yet do not, indicates that the proper stimulus for reproduction is not present at this time. The evidence indeed indicates that the swarming burst is a response to some substance or substances accumulating in the medium during the growth of the colonies and that the latent period is simply an index of the time required for the substance to reach a threshold concentration. That the action of this substance is not through a pH effect has been shown by placing colonies in medium the pH of which has been regulated to that of aged medium and yet the pH-regulated medium has no stimulating effect but parallels normal control medium. The substance is not only heat-stable, but for some reason, has its activity enhanced by high temperature. This increase in activity could conceivably be caused by the removal or breakdown of inhibitory factors present in the medium, or to the production of breakdown products which can further induce colony formation.

The origin of the swarming substance cannot be determined unequivocally from these experiments. The possibility of bacterial origin exists and can only be ruled out through repeat experiments with pure cultures.

It is known, however, that certain algae do produce substances capable of influencing growth. Lefevre, Jacob and Nisbet (1952) in their studies on various fresh water algae have found evidence for the production of substances which can inhibit not only the growth of the algae producing them (autoantagonism) but the growth of other species of algae as well (heteroantagonism). Extensive work with *Pandorina* and *Scenedesmus* has revealed the production by these organisms of inhibitors which the authors have named Pandorinine and Scenedesmine, respectively. These substances, in addition to being autoantagonistic in their inhibitory power, will inhibit the growth of several other algae including various species of *Pediastrum*. It is indeed interesting that boiling the inhibitor usually lowered its inhibitory effect and, in some instances, actually caused it to give an enhancement of growth. This, in some respects, parallels the experiments presented in this paper regarding autoclaving of the swarming substance. It seems quite possible that at least some algae produce both growth inhibitory and stimulating substances as a normal part of their metabolism and that the former are more heat-labile than the latter.

If the swarming substance is produced by the *Pediastrum* colonies then it is most interesting to review the results of Smith (1916) who studied *P. Boryanum* in pure culture. Smith found that for the first two weeks in culture 64- and 32-celled colonies were predominant while a month later 8- and 16-celled colonies were most common. A reasonable explanation of these observations is now available. Since pure cultures in general are prepared with very small inocula it seems likely that Smith's cultures contained small numbers of starting colonies. If growing colonies were producing a swarming inductor it would take a considerable length of time for a small number to produce enough substance to initiate reproduction. The colonies would be expected to grow to a rather large size before a threshold concentration of the substance was reached and colonies with large cell numbers would be produced. However, as the culture grew older new colonies

could not grow as long for the substance would be present tending to induce reproductive activity and colonies of fewer cell number would be produced.

The role of such a substance in the free-living *Pediastrum* colony in nature would be most important. In an actively moving, nutrient-rich environment colonies could grow for an extended time without the substance collecting. In this manner maximum growth would take place and when reproduction finally occurred colonies of great cell number would be produced. In an inactive, stagnant environment, however, the substance would tend to build up and finally force reproduction. It is easy to see the survival value of an increase in numbers for dissemination to more favorable conditions. To speculate even further, it is not difficult to imagine that this swarming substance could, at the proper concentration, stimulate sexual reproduction and the formation of free-swimming gametes. There are some indications that this might be so. It is known, from the author's experience, that when concentrated suspensions of swarming colonies are left standing for a day or more, free-swimming gamete-like cells are often produced. Since fusion between these cells has never been observed it is not known for certain whether they are gametes. Askenasy (1888) found that he could initiate gamete production sooner in his cultures if the colonies were kept in a smaller volume of water. If colonies were living in a drying pond the substance would build up and finally force gamete production. This mechanism would have the advantage of bringing the sexes together in concentrated form, thereby facilitating the meeting of the sexes and fusion. Furthermore, the result of fertilization is a resistant polyhedron which is capable of surviving the drought and germinating again when the rains come and the pond fills up. This hypothesis concerning the role of the swarming substance in sexual reproduction should be capable of testing and experimental work on this subject is planned.

The author wishes to thank Dr. John Tyler Bonner for his fruitful guidance and encouragement during the course of this work.

Summary

1. The characteristics of growth and reproduction in *P. duplex* Meyen have been studied in non-bacteria-free cultures using large inocula of 5 cc. of stock culture for every 50 cc. of fresh medium.

2. It has been found that colonies grown under these conditions exhibit two phases of development: A latent period in which no colony production or swarming takes place but during which the colonies grow, and a period of active swarming or burst phase during which all the cells of the starting colonies give rise to new colonies.

3. It has also been shown that a heat-stable substance is present in aged medium which can, through an effect other than pH, alter the time at which colony production begins, the number of colonies produced and the size of these colonies in terms of cell numbers.

4. It is suggested that the length of the latent period of development is determined by the time required for this substance to reach a threshold concentration. The possible importance of such a substance to free-living forms of *Pediastrum* in nature is also discussed.

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